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The Epstein-Barr Virus Nuclear Protein Encoded by the Leader of the EBNA RNAs Is Important in B-Lymphocyte Transformation

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These experiments evaluate the role of the Epstein-Barr virus (EBV) nuclear antigen leader protein (EBNA-LP) in B-lymphocyte growth transformation by using a recombinant EBV molecular genetic approach. Recombinant viruses encoding for a mutant EBNA-LP lacking the carboxy-terminal 45 amino acids were markedly impaired in their ability to transform primary B lymphocytes compared with EBNA-LP wild-type but otherwise isogenic recombinant viruses. This impairment was particularly evident when primary B lymphocytes were infected under conditions of limiting virus dilution. The impairment could be partially corrected by growth of the infected lymphocytes with fibroblast feeder layers or by cocultivation of primary B lymphocytes with relatively highly permissive mutant virus-infected cells. One of the five mutant recombinants recovered by growth of infected cells on fibroblast feeder cultures was a partial revertant which had a normal transforming phenotype. Several lymphoblastoid cell lines infected with the EBNA-LP mutant recombinant viruses had a high percentage of cells with bright cytoplasmic immunoglobulin staining, as is characteristic of cells undergoing plasmacytoid differentiation. Expression of the other EBV latent or lytic proteins and viral replication were not affected by the EBNA-LP mutations. Thus, the EBNA-LP mutant phenotype is not mediated by an effect on expression of another EBV gene. These data are most compatible with the hypothesis that EBNA-LP affects expression of a B-lymphocyte gene which is a mediator of cell growth or differentiation.

At least nine Epstein-Barr virus (EBV) genes are expressed in latent EBV infection of B lymphocytes (for a review, see reference 21). Two of these genes encode proteins which are expressed earliest in EBV infection, EBV nuclear antigen 2 (EBNA-2) and EBNA leader protein (EBNA-LP) (2). The EBNA-LP RNA contains a downstream EBNA-2 open reading frame, and both EBNA-LP and EBNA-2 can be expressed from this message (37, 47). The EBNA-LP and EBNA-2 RNAs have multiple repeating exons from the EBV genome long internal repeat and three exons from the unique DNA downstream of the repeat. The repeating exons and the first two unique exons encode EBNA-LP and can translate EBNA-LP when one of the two possible acceptor sites for the splice between the first and second exons results in a translational initiation codon at the beginning of the EBNA-LP open reading frame (37). The last exon encodes for EBNA-2 and efficiently translates EBNA-2 from those RNAs which do not translate EBNA-LP because of a splice between the first and second exons which does not result in a translational initiation codon (47). EBNA RNAs expressed after the initial EBNA-LP and EBNA-2 RNAs still have the exons encoding EBNA-LP but sometimes are spliced from upstream of the EBNA-2 open reading frame to exons far downstream which encode EBNA-3A, EBNA-3B, EBNA-3C, or EBNA-1 (6–9, 37, 40). After EBNA-LP and EBNA-2 expression, promoters are activated for RNAs which encode two integral latent membrane proteins (LMPs) (13, 18, 23). The EBNAs and LMPs are likely to be mediators of the EBV latent infection state or of EBV-induced lymphocyte growth transformation (for a review, see reference 21).

Little is as yet known about the functions of EBNA-LP. Since it is encoded in the leader of the EBNA mRNAs, it may be important in regulating EBNA expression or in interacting with EBNAs in the regulation of virus or cell gene expression. The gene to which its expression is most closely coupled is EBNA-2, a transactivator of virus and cell gene expression (46, 48). The EBNA-LP primary amino acid sequence consists mostly of 22- and 66-amino-acid repeat domains which are 25% proline or arginine, 9% serine or threonine, and 6% glutamic acid. The penultimate exon encodes 11 amino acids which are mostly an Arg-Pro repeat. The last exon encodes 34 amino acids which include four arginines and seven terminal acidic residues. EBNA-LP undergoes phosphorylation, probably on the multiple serine and threonine residues, which are in the context of basic amino acids (32). Subnuclear fractionation reveals an association with the nucleoplasmic, chromatin, and nuclear matrix fractions (32). Microscopic analysis with EBNA-LP-specific antibody indicates an association with discrete subnuclear particles (32, 47).

The experiments described here involve a recombinant EBV molecular genetic approach to investigate EBNA-LP's role in B-lymphocyte latent infection or growth transformation. The investigation begins with analyses of the effects of EBNA-LP mutations on the ability of EBV to growth transform B lymphocytes. Previously, in two experiments in which an EBV DNA segment deleted for the last two EBNA-LP exons was recombined into EBV, the mutant recombinant virus yielded fewer cell colonies in a soft agar assay than virus stocks derived when a wild-type DNA segment was recombined into P3HR1 (17). The colonies derived after infection with mutant recombinant virus stocks did not give rise to long-term lymphoblastoid cell lines

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(LCLs) (40a). However, a bulk culture of cells infected with this virus stock grew slowly on a fibroblast feeder layer for several months and eventually grew slowly off fibroblast feeder layers. Analyses of these cells were limited to a Southern blot, which confirmed that the cells were infected with a recombinant EBV containing an EBNA-LP deletion mutation, and an immunoblot, which demonstrated high level EBNA-2 expression in the cells. In similar experiments using an endpoint dilution clonal transformation assay, we had found that primary B lymphocytes infected with mutant recombinant EBV deleted for the last two EBNA-LP exons did not give rise to long-term LCLs. Since the mutant recombinant virus-infected cells in these latter experiments were plated without fibroblast feeder layers, we undertook a series of experiments to determine whether fibroblast feeder layers would support the growth of the mutant recombinant virus-infected cells. The virus mutants and their phenotypic effects on the growth and gene expression of infected primary cells were then characterized.

MATERIALS AND METHODS

Cell lines. The P3HR1 clone 16 (33), W91 (26), IB4 (22), or recombinant EBV-transformed LCLs were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). FS4 is a primary human foreskin fibroblast cell line (kindly provided by J. Vilcek, New York University). HH293 is a primary human placental fibroblast cell line (kindly provided by G. Miller, Yale University).

Plasmids. The *Escherichia coli* plasmid pWT, used to generate recombinant EBVs, was constructed by inserting an EBV DNA W91 *Bgl*II fragment (corresponding to B958 nucleotides 44,664 to 54,359) (5) into the *Bam*HI site of the pHEBo plasmid (42). To make pD1, the last two EBNA-LP exons were deleted from pWT by removing a 350-bp *Hpa*I-*Hin*dIII fragment. Plasmid pS1 was constructed by polymerase chain reaction (PCR) mutagenesis (30). Briefly, part of the EBV W91 *Bam*HI Y fragment was PCR amplified by using primers corresponding to B958 nucleotides 47,744 to 47,788 and 48,284 to 48,304. The first primer contained a 3-bp mutation, changing the first codon of the EBNA-LP Y1 exon from CCA to a TGA stop codon and the second codon from CTA to GTA. The GTA mutation creates a new *Rsa*I site which could readily identify mutant DNA. The second primer included an upstream 20-bp sequence that was not homologous to EBV DNA. The amplified fragment was gel purified and mixed with *Bam*HI-Y DNA. Hybrids between the amplified mutant fragment and the *Bam*HI-Y DNA were then amplified with a third primer corresponding to B958 nucleotides 47,337 to 47,357 and a fourth primer identical to the 20-nucleotide non-EBV sequence in the second primer. The resulting amplified fragment was digested with *Xho*I and *Hin*dIII, and the *Xho*I-*Hin*dIII fragment was used to replace the corresponding fragment of pWT, thus creating pS1. The plasmid pSV*Nae*I*Bam*Z was constructed by inserting the 1.2-kb *Nae*I-*Bam*HI fragment from the *Bam*HI Z fragment of B958 EBV DNA into the *Bam*HI site of the plasmid pSG5 (Stratagene). Plasmid p4IgLP(+) was constructed by subcloning a murine immunoglobulin M (IgM) heavy chain variable region promoter and enhancer from plasmid u/u-gpt (16) into pFR400 (39). A 1.4-kb *Sal*I restriction fragment from u/u-gpt was subcloned by blunt end ligation into the *Pst*I site of pUC-19 to give pIg-19. A 450-bp subfragment of herpes simplex virus type I containing the thymidine kinase polyadenylation signal (19) was cloned into the *Sal*I site in the polylinker of pIg-19 as a *Sac*I-*Hin*dIII fragment giving pIg-19TK. This ligation regenerated the *Sal*I site proximal to the heavy chain promoter and the *Hin*dIII site at the opposite end. Finally, the 1.9-kb *Hin*dIII fragment containing the enhancer-promoter-poly(A) cassette of pIg-19TK was inserted into the unique *Sal*I cloning site of pFR400, with both immunoglobulin and dihydrofolate reductase transcription in the same orientation, to give plasmid p4-Ig. A 1-kb LP cDNA, taken as a *Bam*HI-*Eco*RI I fragment from a pUC-19 subclone was blunt ligated into the *Sal*I cloning site of p4-Ig in a sense orientation [p4IgLP(+)]. p4IgLP(+) was sequenced and had an in-frame fusion between the endogenous immunoglobulin variable region initiation codon and the polylinker sequences involved in the ligation. This results in translation of 17 additional amino acids fused to the amino terminus of EBNA-LP.

Transfections and infections. EBNA-LP mutant or wild-type plasmids (20 μg) were ethanol precipitated with the plasmid pSV*Nae*I*Bam*Z (40 μg), dissolved in 10 μl of sterile water, and transfected into P3HR1 cells with an electroporator (Gene Pulser; Bio-Rad Laboratories) with a pulse of 0.2 V at 960 mF. Three days after transfection, intracellular virus was released by three cycles of freezing and thawing and pooled with the cell supernatant. The pooled material was filtered through a 0.45-μm-pore-size filter, pelleted at 8,800 × *g* for 2 h, and resuspended in 1 ml of complete medium. Each virus preparation was incubated with 10^7 human umbilical cord blood mononuclear cells for 1 h at 37°C and plated in 150 μl of complete medium (RPMI 1640 medium supplemented with 10% FCS and 8 μg of gentamicin per ml) in 64 microtiter wells (with or without fibroblast feeder layers) at 10^6 cells per ml. After 1 week in culture, another 50 μl of complete medium was added to each well. Cultures were then fed weekly with complete medium. Plasmid p4IgLP(+) (75 μg) was transfected into cell lines containing EBNA-LP mutant recombinant virus with an electroporator (Gene Pulsar; Bio-Rad) with a pulse of 0.2 V at 960 mF. After 48 h, the cells were plated in microtiter wells at 100,000 cells per ml and were fed weekly with medium containing 0.25 μM methotrexate sodium (Lederle).

Expansion protocol for EBV-infected cells. When cell growth was evident with visible macroscopic cell clumps and acidified medium, 150 μl of each microtiter well culture was transferred to 1.5 ml of complete medium in a 24-well culture plate (Costar). When growth was evident in the 1.5-ml cultures, the culture volume was increased to 2 ml and then to 5 ml. The 5-ml cultures were transferred to 25-cm^2 tissue culture flasks (Corning). In experiments involving mutant virus-infected cells, cells were seeded in microtiter plates, 24-well plates, and 25-cm^2 tissue culture flasks with and without HH293 or FS4 fibroblast feeder layers.

Passage of virus. Virus was passaged into adult peripheral blood B cells by cocultivating 50,000 irradiated (8,800 rads) virus-transformed cells with 200,000 adult peripheral blood B cells or umbilical cord blood lymphocytes in a 200-μl microwell. Irradiated LCLs and peripheral blood B cells were cultured separately as controls.

PCR amplification of DNA from LCLs. Cells (10^6) were boiled for 15 min in 200 μl of phosphate-buffered saline (160 μg of NaCl–40 μg of KCl–210 μg of Na_2HPO_4–40 μg of KH_2PO_4 per ml) and incubated with 10 μg of proteinase K per ml for 1 h at 65°C. Proteinase K was inactivated by incubation at 100°C for 30 min. One-tenth of this preparation was then PCR amplified (14) with primers corresponding to B958 nucleotides 47,335 to 47,357 and 48,204 to 48,226 (5). PCR conditions were 35 cycles of denaturation at 94°C for 40

s, annealing at 55°C for 1 min, and extension at 72°C for 2 min.

Northern, Southern, and immunoblot analyses. EBNA-1, EBNA-2, EBNA-3, EBNA-LP, and early antigen expression were assayed by immunoblot with EBV-immune human sera. LMP1 and LMP2 were detected by immunoblot with the S12 monoclonal antibody (24) and with an affinity-purified rabbit serum (23), respectively. Immunoglobulin expression was assayed by immunoblot with rabbit anti-human IgM Fc antibody (Jackson Laboratories) and alkaline phosphatase-conjugated goat anti-human IgG (heavy and light chains) antibody (Protoblot). Immunoblots were done as described previously (2). RNA was isolated for Northern blots by acid guanidinium phenol-chloroform extraction (10). RNA was size fractionated on a 1% formaldehyde agarose gel and was transferred to activated nylon membranes (GeneScreen Plus; New England Nuclear). The filters were hybridized with a ^{32}P-labelled EBNA-LP cDNA. For Southern analysis, total cellular DNA digested with *Bam*HI was size fractionated on an 0.8% agarose gel and was transferred to an activated nylon membrane (GeneScreen Plus; New England Nuclear). The filters were hybridized to a ^{32}P-labelled EBV B958 *Bam*HI H fragment.

Immunofluorescence and flow cytometry. Indirect immunofluorescence studies (2) of EBV latent protein expression were done with an EBNA-LP affinity-purified human serum, an EBNA-2 monoclonal antibody (PE2) (50), or an LMP1 monoclonal antibody (S12) (24). Viral replicative protein expression was determined with cells fixed in acetone for 10 min at −20°C and stained with human serum recognizing immediate-early, early, and late viral replicative proteins, a monoclonal antibody, BZ.1, (kindly provided by M. Rowe), or rabbit serum (kindly provided by D. Hayward) to the immediate-early Z protein, the R3 monoclonal antibody to an early replicative protein (31), the L2 monoclonal antibody to the late glycoprotein gp110, or the 2L10 monoclonal antibody to the late glycoprotein gp350 (43). Flow cytometry (46) for cell surface antigen expression was done by using monoclonal antibodies to CD23 (EBVCS1 [36]), CD10 (J5, kindly provided by J. Ritz [34]), ICAM1 (R/R 1/1, kindly provided by T. Springer [35]), LFA1 and LFA3 (TS1/22 and TS2/9, kindly provided by T. Springer [38]), CD39 (AC2, kindly provided by M. Rowe [36]), CD40 (EA-5, kindly provided by T. LeBien), and CD44 (20). Cytoplasmic immunoglobulin staining was determined by cytocentrifugation of cells, fixation in ethanol with 5% acetic acid for 1 h at 4°C, and incubation with fluorescein-conjugated goat anti-human IgG (heavy and light chains) (Jackson Laboratories) for 20 min. Two-color immunofluorescence of EBNA-2 and cytoplasmic immunoglobulin was done with the PE2 monoclonal antibody, fluorescein-conjugated goat anti-mouse antibody, and Texas red-conjugated goat anti-human antibody. Stained cells were visualized with a Zeiss Axioskop equipped for epifluorescence.

Growth curves and dilution studies. Cells were grown to saturation, centrifuged, and resuspended at the desired concentrations in RPMI 1640 with 10% FCS. Cell growth was determined by hemocytometer count or measurement of tritiated thymidine uptake every other day over a 7- to 14-day period. Tritiated thymidine uptake was measured after the cells were pulsed for 16 h with ^{3}H-thymidine (2 μCi/ml). In dilution studies, serial dilutions of EBNA-LP mutant or wild-type virus-transformed cells from the same donor cells were plated in microtiter wells. The cells were observed without additional feeding for the next 6 weeks for evidence of cell growth as manifested by the development of macroscopic clumps of cells and acidification of the medium.

Cell growth in conditioned medium. Cells were taken from the exponentially growing EBNA-LP mutant D1-transformed cell line, resuspended at 10,000 cells per ml in RPMI 1640–10% FCS, and plated into Millicell-CM 0.4-μm-pore-size culture plate inserts (Millipore Corp). The culture plate inserts were placed in 2-ml tissue culture wells (Costar) containing 1 ml of RPMI 1640–10% FCS alone or with 200,000 D1- or WT1-recombinant-transformed LCL cells. The number of viable D1-transformed cells in the culture plate inserts and the number of viable D1- or WT1-transformed cells outside the inserts were determined by hemocytometer count and trypan blue exclusion.

Cytokine production. First-strand cDNA was made from 2.5 μg of LCL cytoplasmic RNA with murine leukemia virus reverse transcriptase (Superscript; Bethesda Research Laboratories) and oligo(dT) primers in 25-μl reactions. Three microliters of the cDNA was then PCR amplified (14) by using primers specific for interleukin-1a (IL-1a), IL-1b, IL-2 through IL-8, tumor necrosis factor-β, and Il-2R (Clontech). PCR conditions were 40 s of denaturation at 94°C, 1 min of annealing at 55°C, and 2 min of extension at 72°C. IL-6 production was determined by using the IL-6-dependent hybridoma cell line B9 (1). B9 cells (2,000) were incubated in 200 μl of serial dilutions of cell-free supernatants from logarithmically growing D1- or WT1-transformed cells. As positive and negative controls, the B9 cells were incubated in RPMI 1640–10% FCS with and without 50 pg of recombinant IL-6 per ml (gift of Genetics Institute). After 84 h in a highly humidified atmosphere of 5% CO_2 at 37°C, the cells were pulsed for 4 h with ^{3}H-thymidine (2 μCi/ml) and harvested (45).

Electron microscopy. Cells were gently pelleted, and the pellet was resuspended in fixative containing 2.5% glutaraldehyde, 2.0% paraformaldehyde, 0.1 M sodium cacodylate, and 1 mM $CaCl_2$ (pH 7.4) for 90 min at 4°C. The cells were then placed in 1% OsO_4 in cacodylate buffer for 1 h at 4°C and stained with 2% aqueous uranyl acetate for 20 min at 4°C. The cell pellets were dehydrated in 35, 70, 95, and then 100% ethanol for 10 min at 4°C, in 100% ethanol twice for 10 min at 25°C, and in propylene oxide twice for 10 min at 25°C. The cell pellets were then infiltrated overnight at room temperature with a 1:1 mix of Epon (Ladd, Inc) and propylene oxide and for 4 h at room temperature in 100% Epon. The pellets were then polymerized overnight at 60°C. Sections (60 nm) from four different areas of each pellet were picked up on 200 mesh (Formvar- and carbon-coated) nickel grids. Sections were poststained with lead citrate and were examined with a Philips electron microscope. Three hundred to five hundred cells of each cell line were viewed.

RESULTS

Effects of EBNA-LP mutations on infected B-lymphocyte outgrowth. EBV recombinants containing mutant or wild-type EBNA-LP were generated by transfecting a cloned EBV DNA fragment into an EBV-infected cell line and inducing virus replication so that the fragment could homologously recombine with replicating viral DNA. The EBV-infected cell line used in these experiments was P3HR1, a Burkitt's lymphoma-derived cell line which contains a replication-competent EBV genome. P3HR1 is deleted for a single EBV DNA segment which includes the last two EBNA-LP-encoding exons and the EBNA-2-encoding exon (Fig. 1) and is, therefore, unable to transform lymphocytes

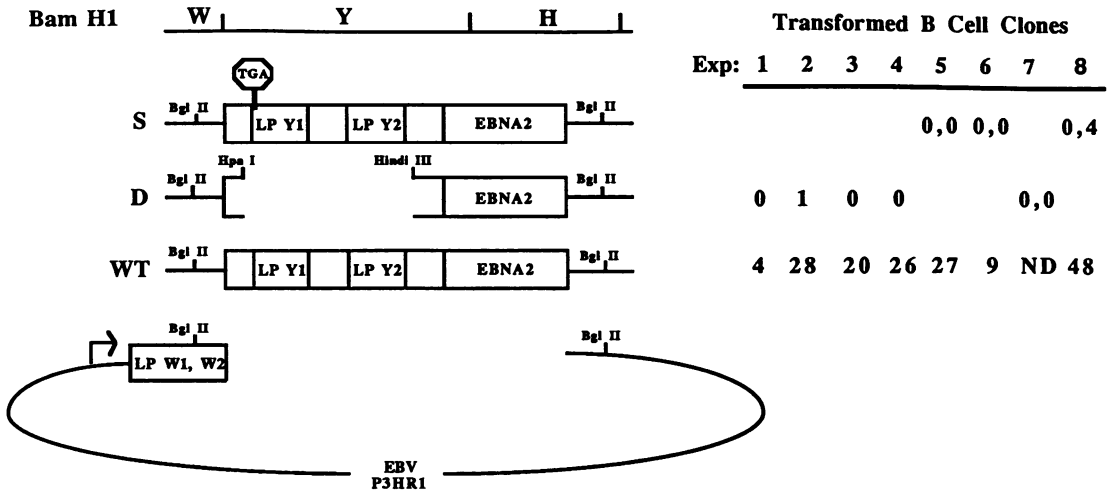

FIG. 1. The P3HR1 EBV genome is deleted for a DNA segment which includes the EBNA-LP Y1 and Y2 exons and the EBNA-2 YH exon and is transformation incompetent. Plasmid pWT containing a wild-type EBV (W91 strain) *Bgl*II DNA fragment which spans the deletion was electroporated into cells to produce transformation-competent EBV recombinants by homologous recombination with replicating P3HR1 DNA. The relevant *Bam*HI map is shown at the top. Cells carrying the P3HR1 EBV genome were induced to replicate by electroporation with a plasmid consisting of the simian virus 40 promoter driving the EBV BZLF1 immediate-early replication cycle transactivator. Plasmid pD1 was constructed from pWT by deletion of a 350-bp *Hpa*I-*Hin*dIII fragment which includes the EBNA-LP Y1 and Y2 exons. Plasmid pS1 has a stop codon at the beginning of the Y1 exon. The virus stocks derived after transfection of each of these plasmids into the P3HR1 cell line were used to infect primary B lymphocytes, and the infected lymphocytes were plated into microwells to allow clonal outgrowth of cells transformed by recombinant viruses. The number of recombinant virus-transformed LCL clones is shown on the right. Each column represents a separate experiment in which 10^7 peripheral blood lymphocytes were infected with wild-type or EBNA-LP mutant (D1 or S1) recombinant virus. In experiment 1, a different electroporator (Pro Genetor; Hoefer) was used and tetradecanoyl phorbol acetate and butyrate, rather than SV*Nae*I*Bam*Z, were used to induce viral replication, which resulted in only four clones of LCLs infected with WT recombinant virus. In experiment 2, a cosmid containing the *Eco*RI A fragment of W91 was used as a positive control rather than pWT. Fifty-five clones were obtained with the *Eco*A cosmid in this experiment. On the basis of the relative efficiency of recombination of pWT compared with the *Eco*A cosmid in several experiments, approximately 28 recombinant EBV clones would be anticipated from pWT in this experiment. In experiment 8, one of the four clones obtained with the EBNA-LP mutant recombinant virus (S1) was found to have mutated the TGA stop codon to TCA and, therefore, encoded an EBNA-LP in which the first two amino acids of the Y1 exon were changed from Pro-Leu to Ser-Val. ND indicates that the wild-type control was not done.

(25, 27). Restoration of the deleted DNA segment by homologous recombination with a transfected EBV DNA fragment can restore the ability of the virus to transform primary B lymphocytes into long-term LCLs (11, 17). Transformation-competent recombinant EBV can then be specifically and clonally recovered by endpoint dilution infection and plating of primary B lymphocytes (11). Mutations can be made in the EBNA-LP- or EBNA-2-encoding exons in the transfected DNA fragment. The effect of the mutations on the ability of the resultant mutant recombinant EBV to growth transform lymphocytes can then be assessed.

In several initial experiments, transformation-competent EBV recombinants were recovered when P3HR1 cells were transfected with a wild-type EBV DNA fragment which encompassed the DNA for which P3HR1 is deleted as well as flanking DNA homologous to P3HR1 (Fig. 1). In contrast, no transformation-competent recombinant virus was recovered after transfection of P3HR1 with an otherwise isogenic EBV DNA fragment deleted for the last two exons of EBNA-LP (Y1 and Y2 in Fig. 1). Since fibroblast feeder layers improve the transformation efficiency of EBV (41), in three additional experiments, primary lymphocytes infected with virus stocks from P3HR1 cells transfected with the EBNA-LP-deleted DNA fragment were plated on fibroblast feeders. In these experiments, only one LCL was obtained (Fig. 1). In contrast, three parallel transfections of P3HR1 with a control wild-type EBV DNA fragment resulted in approximately 20 LCLs per experiment, even in the absence of fibroblast feeder layers (Fig. 1).

Since the Y1 and Y2 exons are part of all EBNA RNAs, recombinant virus with an EBNA-LP deletion could be defective in transforming primary lymphocytes because of a *cis* effect of the deletion on EBNA RNA processing or because the deletion creates an unnatural fusion between the EBNA-LP open reading frame and another coding domain. Therefore, a second mutation in which the first Y1 codon was mutated to a TGA stop codon was evaluated (Fig. 1). This mutation would truncate EBNA-LP at the same place as the deletion mutation but would avoid the *cis* effects of the deletion described above. A single base change was also made in the second Y1 codon, creating a new *Rsa*I site, which could be used to identify cells transformed by EBV recombinants carrying this stop codon mutation. In three separate experiments plated on fibroblast feeder layers, virus stocks derived from transfection of P3HR1 with two independently derived stop codon mutant EBNA-LP DNA fragments gave rise to only four LCLs. All four were from one experiment in which virus stocks derived from transfection of P3HR1 with a wild-type DNA fragment resulted in 48 LCLs in the absence of fibroblast feeders (Fig. 1).

LCLs transformed by EBNA-LP mutant recombinant viruses took longer to be macroscopically visible in microwells with fibroblast feeders than did LCLs transformed by the EBNA-LP wild-type recombinant viruses. After infection of primary B lymphocytes with wild-type recombinant virus, macroscopically visible growth was evident by 4 weeks in approximately 50% of those wells which subsequently yielded LCLs. The remaining 50% had visible

growth by 8 weeks. Outgrowth of the single deletion mutant recombinant virus-transformed LCL was not evident until 5 weeks after infection, while growth of the stop codon mutant virus-transformed LCLs was not evident until 8 to 20 weeks after infection. Cells transformed with wild-type recombinant virus grew in clumps with many viable single cells between the clumped cells, whereas cells transformed by the mutant recombinant viruses had few viable single cells between the clumps.

The initial deletion and stop codon recombinant virus-transformed LCL cultures could not be expanded (see Materials and Methods) without fibroblast feeder layers, except for one stop codon mutant virus-transformed LCL in which the stop codon mutation had reverted (see below). Expansion of the mutant virus-infected LCLs from 200-μl cultures to 1.5-ml cultures in the absence of fibroblast feeder layers resulted in cell death, while most wild-type recombinant virus-infected cells expanded readily under the same conditions. Two stop codon mutant virus-transformed LCLs could never be grown without fibroblast feeder layers. Once the remaining stop codon mutant and the deletion mutant virus-transformed LCLs were growing at high cell density in 2-ml cultures, they were able to grow without fibroblasts when they were maintained at concentrations above approximately 5×10^4 cells per ml. The growth of the deletion mutant-infected cells at concentrations below 5×10^4 cells per ml could be complemented by cocultivation with LCLs growing at high cell concentrations and separated from the deletion mutant-infected cells by a Millicell-CM semipermeable membrane (Millipore Corp.).

The expression of a variety of cell surface antigens known to be affected by EBV infection was analyzed by surface indirect immunofluorescence and flow cytometry. No difference was detected between wild-type and mutant recombinant virus-infected LCLs in the expression of CD10, CD23, ICAM1, LFA1a, LFA3, CD39, CD40, and CD44.

The pool of cells previously transformed by a recombinant EBV (D2), which has a slightly larger EBNA-LP deletion (17) than the deletion described above (D1), was kindly provided by B. Sugden for further characterization in parallel. These cells also grew more slowly than wild-type-infected cells when diluted below approximately 5×10^4 cells per ml.

The mutant- and wild-type-infected LCLs maintained similar viabilities (70% viable after 5 days) when grown in medium supplemented with only 0.1% FCS.

Characterization of the recombinant virus genome. In almost all instances, EBV DNA in the recombinant virus-transformed LCLs had the expected sequence. PCR amplification of an 890-bp sequence which spans the last two EBNA-LP exons gave the expected 890-bp fragment from the wild-type- or stop codon mutant-transformed LCLs, the expected 540-bp fragment from the 350-bp deletion mutant (D1)-transformed LCL, and the expected 390-bp fragment from the 500-bp deletion mutant (D2)-transformed LCL, which was previously derived (17). The fragments amplified from the four stop codon mutant-transformed LCLs all contained the new *Rsa*I site characteristic of that transfected DNA fragment (data not shown).

Although the stop codon mutant virus-transformed LCLs arose in an experiment with an unusually high frequency of wild-type recombinant virus-transformed LCLs, the outgrowth of four mutant virus-transformed LCL clones was surprising, considering only one deletion or stop codon mutant recombinant transformant was obtained in all other experiments. The amplified DNAs, therefore, were sequenced to be certain that they contained the stop codon. The stop codon mutant which grew at early passage without fibroblast feeders was a partial revertant. The TGA stop codon was now TCA so that this recombinant maintained the wild-type open reading frame and differed from the wild type only in having Ser-Val instead of Pro-Leu at the beginning of the Y1-encoded domain. The three other stop codon mutants had the expected TGA replacing the first Y1 codon. The wild-type, deletion, stop codon, and partially revertant recombinant viral genomes were designated WT, D1, S1, and M1, respectively. The previously described deletion mutant (17) was designated D2.

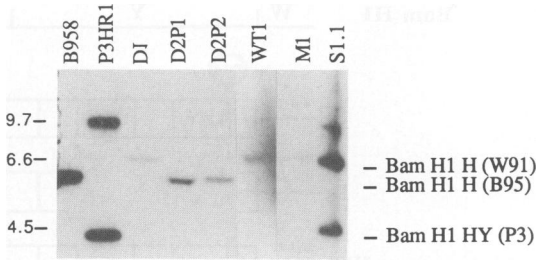

FIG. 2. Southern blot of DNAs from lymphoblastoid cell lines transformed by recombinant EBV produced following electroporation of P3HR1 cells with pWT, pD1, or pS1. D2 was a previously derived recombinant EBV with an EBNA-LP Y1 and Y2 deletion (17). D2P1 and D2P2 were derived from passage of the original D2 virus into primary B cells. M1 was a recombinant EBV derived after pS1 electroporation in which the TGA stop codon had mutated to TCA and produced Ser-Val instead of Pro-Leu at the beginning of the Y1-encoded domain. Also included on the blot was DNA from the P3HR1 Burkitt's lymphoma cell line and from an LCL containing B958 EBV. Total cellular DNA was digested with *Bam*HI and probed with a radiolabeled EBV *Bam*HI H fragment to demonstrate that the recombinant viruses had a *Bam*HI fragment similar in size to that of W91 (6.6 kb) or B958 (6.0 kb) and different from that of the nonrecombinant P3HR1 (4.1 kb) and the transfected plasmid (17 kb). The *Bam*HI H fragment of the D2-transformed cell lines was derived from B958 (17). pWT, pD1, and pS1 were derived from the EBV W91 strain. The band at 9.7 kb seen in some of the lanes corresponds to the EBV DNA *Bam*HI B1 fragment, which has homology to the *Bam*HI H fragment (12). The S1.1 cell line was coinfected with nonrecombinant P3HR1.

Southern blots of *Bam*HI digest of DNA from cell lines transformed by WT, D1, D2, S1, and M1 recombinant EBV were hybridized with a *Bam*HI-H probe and confirmed that homologous recombination had occurred between P3HR1 and the transfected EBV DNA (Fig. 2). Parental P3HR1 EBV DNA has a small 4.1-kb *Bam*HI H hybridizing fragment due to the EBNA-LP and EBNA-2 deletion, which includes all of the *Bam*HI Y fragment and most of *Bam*HI H fragment (Fig. 1 and 2). The transfected plasmid has a 17-kb *Bam*HI H hybridizing fragment because it lacks the *Bam*HI site downstream of the EBNA-2 exon (Fig. 1), and therefore its *Bam*HI H hybridizing fragment includes the 12-kb pHEBo plasmid fused to 5 kb of the EBV *Bam*HI H fragment. The EBV *Bam*HI H DNA fragments in the WT-, D1-, S1-, and M1-transformed LCLs are approximately 6.6 kb, the same size as the *Bam*HI H fragment in the cell line (W91) from which the original transfected EBV DNA fragment was derived (11) (Fig. 2). The EBV *Bam*HI H DNA fragment in the D2-transformed cell lines is 6.0 kb (Fig. 2), identical in size to the *Bam*HI H fragment in the cell line (B958) from which its original transfected EBV DNA fragment was obtained (17). The difference in the sizes of *Bam*HI

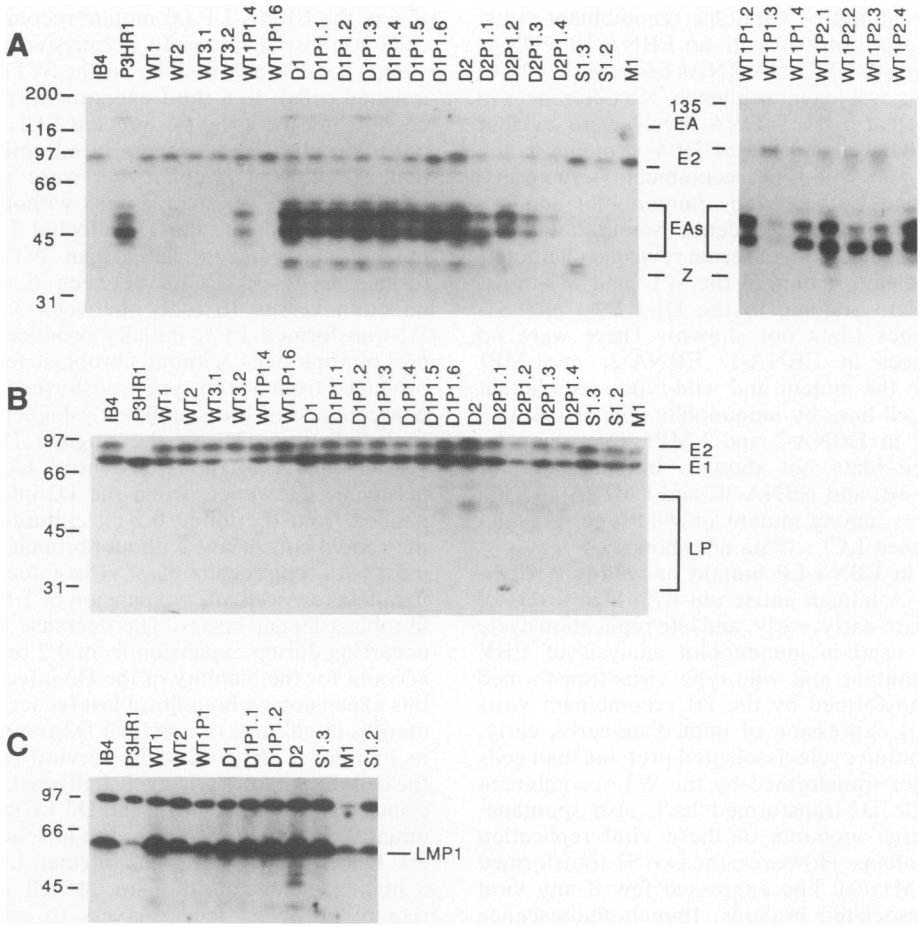

FIG. 3. Immunoblots of EBV proteins in LCLs transformed by EBNA-LP mutant or wild-type recombinant EBV. Proteins from the parental P3HR1 Burkitt's lymphoma cell line and from the latently infected LCL, IB4, are included as controls. The WT1P1.2- to WT1P1.6- and WT1P2.1- to WT1P2.4-infected LCLs are from the same adult donor as the D2P1.1- to D2P1.4-infected LCLs. The D1-infected LCL is from the same donor as the WT1-infected LCL. The WT3.1- and WT3.2-infected LCLs are from the same donor as S1.2-, S1.3-, and M1-infected LCLs. The D1P1.1- to D1P1.6-infected LCLs do not have WT-infected control LCLs from the same donor. (A) Antiserum from an EBV-infected donor detects the 135-kDa early antigen protein, the 40- to 60-kDa early antigen proteins (EA), or Z protein in P3HR1, in seven wild-type recombinants which have been passaged into adult peripheral blood B lymphocytes (WT1P1.2 to WT1P1.4 and WT1P2.1 to WT1P2.4), in the original D1 and D2 EBNA-LP mutant recombinant virus-infected cell lines, as well as in each of the cell lines derived from passage of D1 or D2 into adult peripheral blood B cells (D1P1.1 to D1P1.6 or D2P1.1 to D2P1.4). Little or no early antigen or Z protein expression is seen in IB4, M1, S1.2, S1.3, WT1, WT2, WT3.1, WT3.2, or WT1P1.6. The antiserum also detects EBNA-2 in all LCLs but not in the parental P3HR1 line. (B) Antiserum from an EBV-infected donor shows similar EBNA-2 (E2) and EBNA-1 (E1) levels in the LCLs containing recombinant virus and in the IB4 cell line. The EBNA-LP size varies among the WT cell lines as a consequence of different numbers of internal-repeat-derived domains. (C) Monoclonal antibody to LMP1 shows similar LMP1 levels in LCLs transformed by EBNA-LP mutant or wild-type recombinant virus. P3HR1 cells characteristically express very low levels of LMP1 (29). The bands seen at 97 kDa correspond to a cellular protein which reacts with the S12 antibody. Protein sizes in kilodaltons are indicated on the left.

H DNAs in the W91 and B958 cell lines is probably due to a difference in the number of copies of the internal repeat 2 DNA (12). The band at 9.7 kb seen in some of the lanes corresponds to the *Bam*HI B1 fragment of P3HR1, which is partially homologous to the *Bam*HI H fragment (12). The S1.1 (Fig. 2)-and D2 (data not shown)-transformed cell lines are coinfected with parental, nonrecombinant, P3HR1 EBV and, therefore, contain a small amount of the 4.1-kb parental P3HR1 *Bam*HI YH fragment. However, the D2P1 and D2P2 cell lines, which were infected with recombinant virus from D2-infected cells (see below), contained no detectable parental P3HR1 *Bam*HI YH fragment (Fig. 2). One stop codon mutant-transformed LCL (S1.3) had a *Bam*HI H DNA fragment of approximately 5.3 kb, larger than P3HR1 but smaller than the expected recombinant *Bam*HI H fragment (data not shown). The S1.3 virus probably arose via homologous recombination between the transfected plasmid and P3HR1, because it expressed normal levels of EBNA-2 by immunoblot and immunofluorescence (Fig. 3B). The small size of its *Bam*HI H hybridizing fragment may be due to a spontaneous deletion of part of the *Bam*HI H fragment outside the EBNA-2 open reading frame. This cell line was excluded from further analyses because of the anomalous *Bam*HI H EBV DNA fragment.

Effect of EBNA-LP mutations on EBNA or LMP gene expression. Since EBNA-LP deletions could have *cis* or *trans* effects on EBV gene expression in latently infected, growth-transformed cells, EBV RNA and protein expression in mutant and wild-type recombinant virus-transformed LCLs were compared. Northern blots of cytoplasmic or

nuclear RNA from mutant or wild-type recombinant virus-transformed LCLs were probed with an EBNA-LP cDNA. This probe hybridizes to all EBNA RNAs because they have the EBNA-LP exons as a leader sequence. No differences in cytoplasmic or nuclear EBNA RNA levels were evident (data not shown). The expression of EBV proteins in the EBNA-LP mutant and wild-type recombinant virus-transformed cell lines was compared by immunoblot and immunofluorescence. Immunofluorescence staining with an EBNA-LP affinity-purified human serum revealed characteristic EBNA-LP nuclear staining in the WT- and M1-transformed LCLs and no staining in the D1-, D2-, and S1-transformed cell lines (data not shown). There were no consistent differences in EBNA-1, EBNA-2, or LMP1 expression between the mutant and wild-type recombinant virus-transformed cell lines by immunoblot (Fig. 3B and C) and no differences in EBNA-2 and LMP1 expression by immunofluorescence (data not shown). Immunoblots for EBNA-3A, EBNA-3B, and EBNA-3C and LMP2A also did not show differences among mutant or wild-type recombinant virus-transformed LCLs (data not shown).

EBV replication in EBNA-LP mutant or wild-type virus-transformed LCLs. A human antiserum with high levels of antibody to immediate-early, early, and late replication cycle viral proteins was used in immunoblot analysis of EBV replication in the mutant and wild-type virus-transformed cells. The LCL transformed by the D1 recombinant virus had higher levels of expression of immediate-early, early, and late viral replication cycle-associated proteins than cells from the same donor transformed by the WT recombinant virus (Fig. 3A). The D2-transformed LCL also spontaneously expressed large amounts of these viral replication cycle-associated proteins. However, the two S1-transformed cell lines and the M1 cell line expressed few if any viral replication cycle-associated proteins. Immunofluorescence analysis of EBV replication in the mutant or wild-type virus-transformed LCLs was performed by using the same human antiserum used in the immunoblot analysis or by using the monoclonal antibodies BZ.1, R3, L2, and 2L10 against, respectively, Z immediate-early, BMRF1 early, gp110 late, and gp350 late replication cycle proteins. Approximately 1% of the D1 recombinant EBV-transformed cells, 3% of the D2-recombinant-transformed cells, and less than 0.1% of the WT1- and WT2-transformed cells had positive fluorescence staining with each of the above antibodies. These data indicate that the EBNA-LP Y1 and Y2 exons are not essential for EBV replicative cycle gene expression in LCLs.

Transformation of lymphocytes with EBNA-LP mutant or wild-type recombinant viruses from the original infected LCLs. Since the P3HR1 transfection experiments resulted in only a limited number of mutant recombinant virus-infected LCLs in which the phenotypic effects of the mutations could be studied, attempts were made to derive new LCLs by passage of the initial recombinant viruses to primary lymphocytes. Larger numbers of clones transformed by these mutant viruses could better control for cell-specific effects and allow more accurate delineation of the mutant EBNA-LP phenotype. To passage the recombinants into new cells, the original LCLs were lethally irradiated with 8,800 rads and cocultivated with adult peripheral blood B cells or with umbilical cord blood lymphocytes. Two attempts to transform cells with the D1 virus without fibroblast feeder layers were unsuccessful, despite the ready passage in parallel experiments of the WT1 wild-type recombinant virus and greater virus replication in the D1-infected LCL (1% of the EBNA-LP D1 mutant recombinant virus-infected cells were spontaneously permissive for virus replication versus <0.1% permissivity in the WT1 recombinant virus-infected cells). In a third experiment, primary lymphocytes cocultivated with the D1-infected LCL were plated directly onto fibroblast feeder layers in microtiter wells. The same donor's primary lymphocytes were also cocultivated in parallel with WT1-infected cells without fibroblast feeders. In this experiment, the D1-infected LCLs were fewer in number and came up later than WT1 secondarily transformed LCLs (3 LCLs between 6 and 12 weeks after infection versus 16 LCLs between 3 and 6 weeks). The D2-transformed LCL initially produced as many wells of proliferating cells without fibroblast feeder layers as were produced from a wild-type-transformed LCL, probably as a consequence of the significantly higher level of productive virus infection (3% in the original D2-transformed LCL versus <0.1% in the WT control LCL used in this experiment). However, when the D2-infected cells were expanded from the initial 0.2-ml cultures to 1.5-ml cultures, they could not survive without fibroblast feeder layers, while most wild-type recombinant virus-infected LCLs derived in parallel grew well after expansion to 1.5-ml cultures without fibroblast feeder layers. The decrease in cell concentration occurring during expansion from 0.2 to 1.5-ml cultures may account for the inability of the D2-infected LCLs to survive this expansion without fibroblast feeder layers. After several months in culture, the original D2-transformed cell line lost its high level of permissivity to viral replication (<0.1% of the cells expressed lytic cycle antigens), and cocultivation of primary lymphocytes with the D2 LCL no longer produced initially proliferating cells in the absence of fibroblast feeders. Once the new D1- or D2-infected LCLs were growing at a high cell concentration in 24-well plates, they did not require fibroblast feeder layers to continue growing and (unlike the original D1- and D2-transformed LCLs) grew as well as the wild-type-infected LCLs, even at low cell concentrations. The original M1 virus-transformed LCL was not detectably permissive for virus replication, and only 2 clones were obtained from passage of the M1 revertant recombinant virus without fibroblast feeders versus 14 clones from a wild-type recombinant virus passaged in parallel. The M1-virus-transformed LCLs were transferred from the initial microtiter wells to 1.5-ml wells without fibroblast feeder layers and grew as readily as the wild-type recombinant virus-infected controls. Thus, the new deletion mutant recombinant virus-infected LCLs all required fibroblast feeders for survival through our expansion protocol (as described in Materials and Methods), whereas the M1 two-amino-acid substitution revertant and the wild-type control transformed cells did not require fibroblast feeders. Attempts to passage the S1.1 and S1.2 mutant recombinant viruses, even with fibroblast feeder layers, were unsuccessful, although a WT recombinant virus from a cell line with a similarly low level of virus production passaged in parallel yielded multiple transformed cell wells. The difficulty in passaging the S1 recombinants is likely to be due to the combined effects of the low level (<0.1%) of spontaneous virus replication in the two original S1-transformed LCLs, the poor growth of the original S1-infected cell lines, which markedly restricted the number of cocultivation experiments which could be performed, and the S1 mutation effect on primary lymphocyte growth transformation.

Immunoblot and immunofluorescence analyses of EBV gene expression in the LCLs infected with passaged recombinant virus revealed no consistent differences in EBNA-1,

EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, LMP1, or LMP2a expression by immunoblot (Fig. 3), as was characteristic of the initial LCLs transformed by the D1, D2, S1, and M1 recombinants. Although the initial D1- and D2-transformed LCLs were more permissive for viral replication than were the initial WT-transformed LCLs, no difference in permissivity to viral replication was noted by immunofluorescence or immunoblot when LCLs into which the D1, D2, and M1 viruses were passaged were compared with LCLs from the same donor into which WT virus was passaged (Fig. 3A and Table 1). Therefore, the higher level of permissivity to viral replication seen in the initial D1- or D2-infected LCLs compared with that in the wild-type-transformed controls was probably due to cell-specific factors rather than to the EBNA-LP deletions.

The decreased transformation efficiency of the EBNA-LP deletion and stop codon mutant recombinant viruses was not due to a lower virus yield from the mutant virus-transformed cells. As noted above, these cells were at least as permissive for replicative cycle antigen expression by immunofluorescence and immunoblot. By electron microscopic analysis, virus morphogenesis and viral particle number in productively infected cells did not differ significantly between the deletion mutant and wild-type recombinant virus-transformed LCLs. To evaluate the relative amounts of virus and transforming activity in the EBNA-LP mutant versus wild-type virus-infected cells, virus replication and transforming activity were assayed in two D1- and two WT recombinant virus-transformed cell lines which had been derived in parallel by infection of the same donor's lymphocytes. The two D1-infected cell lines produced no transforming virus as assayed by cocultivation with primary B lymphocytes without fibroblast feeder layers. One to two percent of the cells in these mutant virus-infected LCLs were spontaneously permissive for virus replication by immunofluorescence and electron microscopic analysis. Both wild-type control virus-infected cells did produce transforming virus in the same assay when less than 0.1% of the cells in one cell line and 1% of the cells in the other cell line were spontaneously permissive for virus replication by immunofluorescence and electron microscopy. These data confirm that the EBNA-LP deletion mutant recombinant virus is less efficient at transformation than the wild-type virus.

The effect of EBNA-LP mutations on B-lymphocyte differentiation. Since plasmacytoid differentiation with increased cytoplasmic immunoglobulin expression has been associated with decreased B-lymphocyte replicative capacity (49), cytoplasmic immunoglobulin levels in the mutant and wild-type virus-transformed cell lines were compared. Most D1- or D2-transformed cell lines and all M1- and WT-transformed cell lines had bright cytoplasmic immunoglobulin fluorescence staining in less than 5% of the cells after several months in culture (Fig. 4). However, two of nine D1-transformed, one of five D2-transformed, and two of two S1-transformed LCLs had bright cytoplasmic immunoglobulin staining in 10 to 40% of the cells, even after several months in culture (Fig. 4 and Table 1). Total cellular immunoglobulin levels by immunoblot did not differ among cell lines with or without high level immunoglobulin staining (data not shown). EBV-infected cells with bright cytoplasmic immunoglobulin staining have been previously noted to have decreased EBNA expression and decreased replicative capacity (49). In the EBNA-LP mutant virus-infected cell lines, the intensity of cytoplasmic immunoglobulin staining was also inversely correlated with the intensity of EBNA-2 staining by two-color immunofluorescence (data not shown).

TABLE 1. Comparison of fibroblast dependence, permissivity for viral replication, and cytoplasmic immunoglobulin staining between mutant and wild-type recombinant virus-transformed LCLs

LCL[a]	Fibroblast dependence[b]	Cells permissive for viral replication (%)[c]	Cells with cytoplasmic Ig staining (%)
WT1	−	<1	<1
D1	+	3	<1
WT3.1	−	<1	<1
WT3.2	−	<1	<1
M1	−	<1	<1
S1.1	+	<1	37
S1.2	+	<1	23
WT1P1.1	−	<1	<1
WT1P1.2	−	3	<1
WT1P1.3	−	<1	5
WT1P1.4	−	<1	5
WT1P1.5	−	2	<1
D2P1.1	+	1	7
D2P1.2	+	<1	29
D2P1.3	+	<1	2
D2P1.4	+	2	8
WT1P2.1	−	<1	6
WT1P2.2	−	<1	5
WT1P2.3	−	4	5
WT1P2.4	−	5	5
D1P2.1	+	5	8
D1P2.2	+	1	3
D1P2.3	+	8	31
D1P1.1	+	3	11
D1P1.2	+	4	<1
D1P1.3	+	1	<1
D1P1.4	+	7	<1
D1P1.5	+	1	<1
WT3.1P1.1	−	<1	<1
WT3.1P1.2	−	<1	<1
M1P1.1	−	<1	<1
M1P1.2	−	<1	<1

[a] Mutant and wild-type virus-infected cells from the same donor are grouped together. The D1P1 cell lines did not have WT-transformed control cell lines from the same donor. WT1P1.1 to WT1P1.4 are four cell lines established from one passage of the WT1 virus into adult peripheral blood B lymphocytes. WT1P2.1 to WT1P2.4 are LCLs established from a second passage of the same virus into cells from a different donor. WT3.1P1.1 and WT3.1P1.2 are two cell lines established from one passage of the WT3.1 virus into umbilical cord blood lymphocytes. M1P1.1 and M1P1.2 are two cell lines established from passage of the M1 virus into umbilical cord blood lymphocytes. D1P1.1 to D1P1.5 and D1P2.1 to D1P2.3 represent passages of the D1 virus to B lymphocytes from two different adult donors. The WT1P2.1 to WT1P2.4 and D1P2.1 and D1P2.3 LCLs were studied 5 to 6 weeks after infection. The other LCLs were studied 6 to 12 months after infection.

[b] Fibroblast dependence, the dependence of LCLs on fibroblast feeders for growth.

[c] The percentage of cells permissive for viral replication as determined by immunofluorescence staining with the BZ.1 monoclonal antibody.

Cytokine production. Differences between mutant and wild-type LCLs in fibroblast feeder dependence and cytoplasmic immunoglobulin staining could be due to an effect of EBNA-LP on cytokine production. Cytokine mRNAs in the D1 and wild-type recombinant virus-transformed cell lines were qualitatively compared by PCR amplification of cDNA. PCR with cytokine-specific probes (Clontech) revealed IL-1a, IL-1b, IL-2 through IL-8, tumor necrosis factor-β, and IL-2R expression in both the D1 and wild-type recombinant

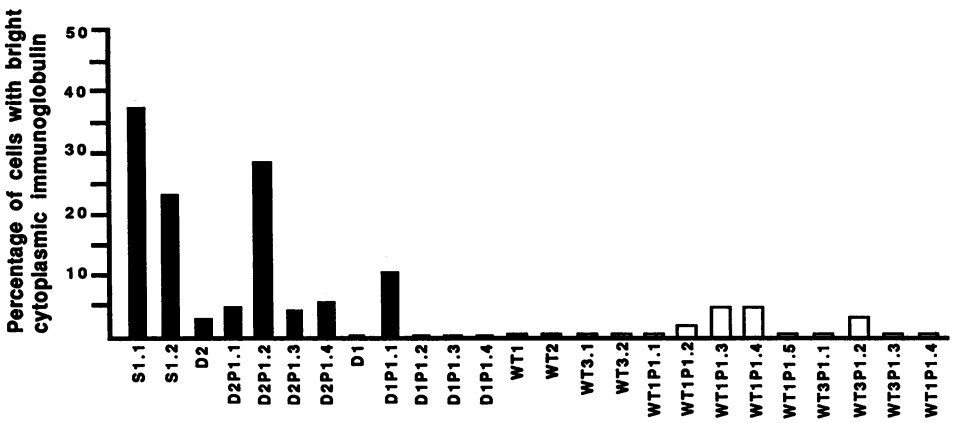

FIG. 4. Percentages of cells in LCLs transformed by EBNA-LP mutant or wild-type recombinant virus which express high levels of cytoplasmic immunoglobulin. All LCLs in Table 1 were studied at least 6 months after EBV infection. Cells were cytocentrifuged, fixed in ethanol and acetic acid, and stained with a fluorescein-conjugated goat anti-human IgG (heavy and light chains). The percentages of mutant (black boxes) or wild-type (white boxes) recombinant virus-transformed cells with bright cytoplasmic immunoglobulin staining are shown. The lymphocyte donor for each of the mutant and wild-type cell lines is described in footnote *a* of Table 1.

virus-transformed cell lines. This does not exclude the possibility of quantitative differences in cytokine production. Since IL-6 is an autocrine growth factor for EBV-infected cells (45), IL-6 production by the D1 and WT1 recombinant virus-transformed cell lines was assayed more quantitatively by measuring the growth of the IL-6-dependent B9 cell line grown in the presence of supernatants from D1- or WT1-infected cells. Both the deletion mutant and the wild-type virus-transformed cell lines made similarly low levels of IL-6. Further, 1.6 ng of recombinant IL-6 per ml (Genetics Institute) minimally enhanced the growth rate of the original D1-transformed LCL (data not shown).

The effect of wild-type EBNA-LP expression in EBNA-LP deletion mutant recombinant virus-transformed cells. A positive selection expression plasmid for EBNA-LP was transfected into the D2-transformed LCL to evaluate the effects of wild-type EBNA-LP expression in this mutant recombinant virus-transformed LCL. Transfected cells were selected without fibroblast feeder layers. Three methotrexate-resistant clones were obtained. All three clones had characteristic EBNA-LP nuclear immunofluorescence staining with an EBNA-LP affinity-purified human serum which does not react with the deleted EBNA-LPs. However, the intensity of the fluorescence staining was weaker than in wild-type recombinant-transformed LCLs. PCR amplification with primers specific for the transfected plasmid confirmed that the three clones contained the transfected plasmid. No change in EBNA-1, EBNA-2, or viral replication protein expression was noted by immunofluorescence staining or immunoblot of the EBNA-LP expressing clones compared with that of the parental D2-transformed LCL. As was characteristic of the parental LCL, the EBNA-LP-positive clones could not grow when they were seeded at less than 5×10^4 cells per ml, despite the presence of EBNA-LP. The failure of wild-type EBNA-LP to complement this abnormal growth phenotype could be due to inadequate EBNA-LP expression, to a transdominant effect of the EBNA-LP mutant, or to the abnormal growth phenotype being cell rather than virus specific. The fact that other LCLs infected with passaged D2 or D1 mutant recombinant virus had normal growth rates at low cell concentrations is consistent with the latter possibility.

DISCUSSION

These experiments indicate that the EBNA-LP is critical for B-lymphocyte transformation under the usual conditions applied to assay EBV-transforming activity. In the absence of fibroblast feeders, no LCLs were recovered after infection of B lymphocytes with mutant EBNA-LP recombinant virus from transfected P3HR1 cells. Even in the presence of fibroblast feeders, LCLs were recovered only rarely after infection of B lymphocytes with mutant EBNA-LP recombinant virus from transfected P3HR1 cells. In contrast, many LCLs were recovered in the absence of fibroblast feeders after infections with otherwise isogenic wild-type recombinant virus from P3HR1 cells transfected in parallel. EBNA-LP stop codon mutant-infected cells were at least as impaired in their outgrowth as the deletion mutant-infected cells, indicating that the effect on LCL outgrowth is due to the carboxy-terminal 45-amino-acid truncation of the EBNA-LP.

The stop codon mutation offers the least ambiguous opportunity to ascertain the role of the last two EBNA-LP exons in cell growth transformation. The very low number of stop codon mutant recombinant virus-transformed LCLs and the persistently poor growth of these LCLs, even on fibroblast feeders, indicate that the last 45 amino acids are critical to full growth transformation of primary B lymphocytes. Importantly, the use of two independently derived stop codon mutant plasmids for the generation of these EBV recombinants and the nearly normal B-lymphocyte transforming activity of the M1 revertant from the stop codon transfections exclude the unlikely possibility that the stop codon recombinant DNAs have a spontaneous mutation elsewhere which results in the altered growth transformation phenotype. The occurrence of a revertant is a rare genetic event, and its appearance in one of four LCLs arising from the stop codon transfections suggests that there is a tremendous selective advantage in our transformation assays for outgrowth of cells infected with virus which encodes the last 45 amino acids of the EBNA-LP. The persistently poor growth of the LCLs transformed by the stop codon recombinants, even on fibroblast feeder layers, and their lack of

permissivity to virus replication have so far restricted extensive cellular or molecular biologic analyses.

Although the last two exons of the EBNA-LP are important in primary B-lymphocyte growth transformation, they are not absolutely essential. Primary B lymphocytes infected with EBNA-LP mutant recombinant EBV infrequently could be recovered as LCLs when they were maintained on fibroblast feeder layers or when the primary B lymphocytes were cocultivated with a very productive deletion mutant-virus-infected LCL. Presumably, cell outgrowth under the latter conditions resulted from many simultaneously initiated transforming events. However, the growth of these infected primary cells could not be maintained without fibroblast feeder support when the cultures were initially expanded. Thus, the phenotype of the deletion of the last 45 amino acids of EBNA-LP was profoundly evident when primary B lymphocytes were infected in an endpoint dilution clonal transformation assay without fibroblast feeders by using recombinant virus from transfected P3HR1 cells, slightly less evident when the infected cells were plated with fibroblast feeders, and consistently evident when mutant virus-infected cells were initially expanded from outgrowth in 96-well microtiter plates to 24-well plates.

Although wild-type EBV infection readily causes indefinite B-lymphocyte proliferation without a stringent requirement for fibroblast feeders, fibroblast feeders improve transformation efficiency (41). A likely hypothesis which could explain the fibroblast feeder effect in primary B-lymphocyte growth transformation by wild-type EBV is that fibroblasts provide growth factor(s) which positively affect the probability that any EBV-infected B lymphocyte will give rise to an LCL. The unusual dependence of EBNA-LP mutant recombinant EBV-infected cells on fibroblast feeders could be due to an adverse effect of the EBNA-LP mutation on the ability of the recombinant EBV to induce an autocrine growth factor pathway necessary for full B-lymphocyte growth transformation and complementation of the defect by fibroblast derived growth factors. Since IL-6 is a fibroblast factor which positively affects EBV-infected cell outgrowth (45), IL-6 production was evaluated in the EBNA-LP D1 mutant recombinant LCL. No difference in IL-6 production was detected between the D1 and WT1 recombinant virus-infected cell lines. Furthermore, recombinant IL-6 had little effect on EBNA-LP D1 recombinant virus-infected cell growth.

An alternative possibility is that the last two EBNA-LP exons could be more stringently required for the transformation of most B lymphocytes than they are for transformation of a less common B-cell type which can be transformed by the mutant recombinant EBV as long as the cells are initially supported by fibroblast feeders. Little is known about whether fibroblast feeder layers differentially facilitate the outgrowth of specific B-lymphocyte subsets after EBV infection. Small resting B lymphocytes more readily give rise to LCLs after infection with wild-type EBV (3), and EBV-transformed LCLs arise almost exclusively from those B lymphocytes which express surface CD23 after EBV infection (4, 44). However, EBNA-LP D1 mutant recombinant virus-transformed cells did not differ from WT recombinants in CD23 or other surface activation or adhesion molecule expression.

Although cells infected with the EBNA-LP D1 deletion mutant recombinant virus do not differ from cells infected with a wild-type recombinant in their expression of surface markers characteristic of EBV-transformed LCLs, some of the mutant recombinant virus-transformed LCLs differ from wild-type recombinant virus-transformed LCLs in plasmacytoid differentiation, a phenotype which has been linked to specific B-lymphocyte populations and to decreased B-lymphocyte proliferative capacity (15, 24a, 49). It is possible that EBNA-LP plays an indirect role in suppressing terminal B-cell differentiation in EBV-infected cells.

These experiments do not identify the specific biochemical mechanisms for EBNA-LP's role in cell growth transformation. However, the lack of effect of the mutations on expression of other EBNAs or LMPs makes it unlikely that EBNA-LP is involved in the complex processing of EBV RNAs, as might be inferred from its association with small subnuclear particles. Complementation of the EBNA-LP mutation-related defect in LCL outgrowth by fibroblast feeders favors the hypothesis that EBNA-LP regulates expression of a cell growth factor. The last two EBNA-LP exons have acidic and proline- and arginine-rich domains, as have been identified in some transcriptional regulatory molecules (28). The molecular genetic experiments reported here delineate a stringent, albeit not absolute, requirement for EBNA-LP in LCL outgrowth. The assay conditions described here can be pursued to define more precisely the domains encoded by the last two EBNA-LP exons, which are critical for LCL outgrowth.

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REFERENCES

1. **Aarden, L. A., G. E. De, O. L. Schaap, and P. M. Lansdorp.** 1987. Production of hybridoma growth factor by human monocytes. Eur. J. Immunol. **17**:1411–1416.
2. **Alfieri, C., M. Birkenbach, and E. Kieff.** 1991. Early events in Epstein-Barr virus infection of human B lymphocytes. Virology **181**:595–608.
3. **Aman, P., B. Ehlin-Henriksson, and G. Klein.** 1984. Epstein-Barr virus susceptibility of normal human B lymphocyte populations. J. Exp. Med. **159**:208–220.
4. **Azim, T., and D. H. Crawford.** 1988. Lymphocytes activated by the Epstein-Barr virus to produce immunoglobulin do not express CD23 or become immortalized. Int. J. Cancer **42**:23–28.
5. **Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Seguin et al.** 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature (London) **310**:207–211.
6. **Bodescot, M., O. Brison, and M. Perricaudet.** 1986. An Epstein-Barr virus transcription unit is at least 84 kilobases long. Nucleic Acids Res. **14**:2611–2620.
7. **Bodescot, M., B. Chambraud, P. Farrell, and M. Perricaudet.** 1984. Spliced RNA from the IR1-U2 region of Epstein-Barr virus: presence of an open reading frame for a repetitive polypeptide. EMBO J. **3**:1913–1917.
8. **Bodescot, M., and M. Perricaudet.** 1986. Epstein-Barr virus mRNAs produced by alternative splicing. Nucleic Acids Res. **14**:7103–7114.
9. **Bodescot, M., and M. Perricaudet.** 1987. Clustered alternative splice sites in Epstein-Barr virus RNAs. Nucleic Acids Res. **15**:5887.
10. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J.**

Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.

11. **Cohen, J. I., F. Wang, J. Mannick, and E. Kieff.** 1989. Epstein-Barr virus nuclear protein 2 is a key determinant of lymphocyte transformation. Proc. Natl. Acad. Sci. USA **86**:9558–9562.
12. **Dambaugh, T., and E. Kieff.** 1982. Two related tandem direct repeat sequences in EBV DNA. J. Virol. **44**:823–833.
13. **Fennewald, S., S. van Santen, and E. Kieff.** 1984. Nucleotide sequence of an mRNA transcribed in latent growth-transforming virus infection indicates that it may encode a membrane protein. J. Virol. **51**:411–419.
14. **Frohman, M. A., M. K. Dush, and G. R. Martin.** 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA **85**:8998–9002.
15. **Gregory, C. D., C. Kirchgens, C. F. Edwards, L. S. Young, M. Rowe, A. Forster, T. H. Rabbitts, and A. B. Rickinson.** 1987. Epstein-Barr virus-transformed human precursor B cell lines: altered growth phenotype of lines with germ-line or rearranged but nonexpressed heavy chain genes. Eur. J. Immunol. **17**:1199–1207.
16. **Grosschedl, R., and D. Baltimore.** 1985. Cell-type specificity of immunoglobulin gene expression is regulated by at least three DNA sequence elements. Cell **41**:885–897.
17. **Hammerschmidt, W., and B. Sugden.** 1989. Genetic analysis of immortalizing functions of Epstein-Barr virus in human B lymphocytes. Nature (London) **340**:393–397.
18. **Hennessy, K., S. Fennewald, M. Hummel, T. Cole, and E. Kieff.** 1984. A membrane protein encoded by Epstein-Barr virus in latent growth-transforming infection. Proc. Natl. Acad. Sci. USA **81**:7207–7211.
19. **Hubenthal, V. J., R. A. Houghten, L. Pereira, and B. Roizman.** 1988. Mapping of functional and antigenic domains of the alpha 4 protein of herpes simplex virus 1. J. Virol. **62**:454–462.
20. **Jalkanen, S. T., R. F. Bargatze, L. R. Herron, and E. C. Butcher.** 1986. A lymphoid cell surface glycoprotein involved in endothelial cell recognition and lymphocyte homing in man. Eur. J. Immunol. **16**:1195–1202.
21. **Kieff, E., and D. Liebowitz.** 1990. Epstein-Barr virus and its replication, p. 1889–1920. *In* B. N. Fields and D. M. Knipe (ed.), Virology. Raven Press, Ltd., New York.
22. **King, W., A. L. Thomas-Powell, N. Raab-Traub, M. Hawke, and E. Kieff.** 1980. Epstein-Barr virus RNA. V. Viral RNA in a restringently infected, growth-transformed cell line. J. Virol. **36**:506–518.
23. **Longnecker, R., and E. Kieff.** 1990. A second Epstein-Barr virus membrane protein (LMP2) is expressed in latent infection and colocalizes with LMP1. J. Virol. **64**:2319–2326.
24. **Mann, K. P., D. Staunton, and L. D. Thorley.** 1985. Epstein-Barr virus-encoded protein found in plasma membranes of transformed cells. J. Virol. **55**:710–720.

24a. **Melamed, M. D., J. Gordon, S. J. Ley, D. Edgar, and J. N. C. Hughes.** 1985. Senescence of a human lymphoblastoid clone producing anti-rhesus(D). Eur. J. Immunol. **15**:742–746.

25. **Menezes, J., W. Leibold, and G. Klein.** 1975. Biological differences between Epstein-Barr virus (EBV) strains with regard to lymphocyte transforming ability, superinfection and antigen induction. Exp. Cell Res. **92**:478–484.
26. **Miller, G., D. Coope, J. Niederman, and J. Pagano.** 1976. Biological properties and viral surface antigens of Burkitt lymphoma- and mononucleosis-derived strains of Epstein-Barr virus released from transformed marmoset cells. J. Virol. **18**: 1071–1080.
27. **Miller, G., J. Robinson, L. Heston, and M. Lipman.** 1974. Differences between laboratory strains of Epstein-Barr virus based on immortalization, abortive infection, and interference. Proc. Natl. Acad. Sci. USA **71**:4006–4010.
28. **Mitchell, P. J., and R. Tjian.** 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245**:371–378.
29. **Murray, R. J., L. S. Young, A. Calender, C. D. Gregory, M. Rowe, G. M. Lenoir, and A. B. Rickinson.** 1988. Different patterns of Epstein-Barr virus gene expression and of cytotoxic T-cell recognition in B-cell lines infected with transforming (B95.8) or nontransforming (P3HR1) virus strains. J. Virol. **62**:894–901.
30. **Nelson, R. M., and G. L. Long.** 1989. A general method of site-specific mutagenesis using a modification of the Thermus aquaticus polymerase chain reaction. Anal. Biochem. **180**:147–151.
31. **Pearson, G. R., J. Luka, L. Petti, J. Sample, M. Birkenbach, D. Braun, and E. Kieff.** 1987. Identification of an Epstein-Barr virus early gene encoding a second component of the restricted early antigen complex. Virology **160**:151–161.
32. **Petti, L., C. Sample, and E. Kieff.** 1990. Subnuclear localization and phosphorylation of Epstein-Barr virus latent infection nuclear proteins. Virology **176**:563–574.
33. **Rabson, M., L. Gradoville, L. Heston, and G. Miller.** 1982. Non-immortalizing P3J-HR-1 Epstein-Barr virus: a deletion mutant of its transforming parent, Jijoye. J. Virol. **44**:834–844.
34. **Ritz, J., J. M. Pesando, M. J. Notis, H. Lazarus, and S. F. Schlossman.** 1980. A monoclonal antibody to human acute lymphoblastic leukaemia antigen. Nature (London) **283**:583–585.
35. **Rothlein, R., M. L. Dustin, S. D. Marlin, and T. A. Springer.** 1986. A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. J. Immunol. **137**:1270–1274.
36. **Rowe, M., J. E. Hildreth, A. B. Rickinson, and M. A. Epstein.** 1982. Monoclonal antibodies to Epstein-Barr virus-induced, transformation-associated cell surface antigens: binding patterns and effect upon virus-specific T-cell cytotoxicity. Int. J. Cancer **29**:373–381.
37. **Sample, J., M. Hummel, D. Braun, M. Birkenbach, and E. Kieff.** 1986. Nucleotide sequences of mRNAs encoding Epstein-Barr virus nuclear proteins: a probable transcriptional initiation site. Proc. Natl. Acad. Sci. USA **83**:5096–5100.
38. **Sanchez, M. F., A. M. Krensky, C. F. Ware, E. Robbins, J. L. Strominger, S. J. Burakoff, and T. A. Springer.** 1982. Three distinct antigens associated with human T-lymphocyte-mediated cytolysis: LFA-1, LFA-2, and LFA-3. Proc. Natl. Acad. Sci. USA **79**:7489–7493.
39. **Simonsen, C. C., and A. D. Levinson.** 1983. Isolation and expression of an altered mouse dihydrofolate reductase cDNA. Proc. Natl. Acad. Sci. USA **80**:2495–2499.
40. **Speck, S. H., and J. L. Strominger.** 1985. Analysis of the transcript encoding the latent Epstein-Barr virus nuclear antigen I: a potentially polycistronic message generated by long-range splicing of several exons. Proc. Natl. Acad. Sci. USA **82**:8305–8309.

40a. **Sugden, B.** Personal communication.

41. **Sugden, B., and W. Mark.** 1977. Clonal transformation of adult human leukocytes by Epstein-Barr virus. J. Virol. **23**:503–508.
42. **Sugden, B., K. Marsh, and J. Yates.** 1985. A vector that replicates as a plasmid and can be efficiently selected in B-lymphoblasts transformed by Epstein-Barr virus. Mol. Cell. Biol. **5**:410–413.
43. **Tanner, J., J. Weis, D. Fearon, Y. Whang, and E. Kieff.** 1987. Epstein-Barr virus gp350/220 binding to the B lymphocyte C3d receptor mediates adsorption, capping, and endocytosis. Cell **50**:203–213.
44. **Thorley-Lawson, D. A., and K. P. Mann.** 1985. Early events in Epstein-Barr virus infection provide a model for B cell activation. J. Exp. Med. **162**:45–59.
45. **Tosato, G., J. Tanner, K. D. Jones, M. Revel, and S. E. Pike.** 1990. Identification of interleukin-6 as an autocrine growth factor for Epstein-Barr virus-immortalized B cells. J. Virol. **64**:3033–3041.
46. **Wang, F., C. D. Gregory, M. Rowe, A. B. Rickinson, D. Wang, M. Birkenbach, H. Kikutani, T. Kishimoto, and E. Kieff.** 1987. Epstein-Barr virus nuclear antigen 2 specifically induces expression of the B-cell activation antigen CD23. Proc. Natl. Acad. Sci. USA **84**:3452–3456.
47. **Wang, F., L. Petti, D. Braun, S. Seung, and E. Kieff.** 1987. A bicistronic Epstein-Barr virus mRNA encodes two nuclear

proteins in latently infected, growth-transformed lymphocytes. J. Virol. **61**:945–954.

48. **Wang, F., S. F. Tsang, M. G. Kurilla, J. I. Cohen, and E. Kieff.** 1990. Epstein-Barr virus nuclear antigen 2 transactivates latent membrane protein LMP1. J. Virol. **64**:3407–3416.
49. **Wendel-Hansen, V., A. Rosen, and G. Klein.** 1987. EBV-transformed lymphoblastoid cell lines down-regulate EBNA in parallel with secretory differentiation. Int. J. Cancer **39**:404–408.
50. **Young, L., C. Alfieri, K. Hennessy, H. Evans, C. O'Hara, K. C. Anderson, J. Ritz, R. S. Shapiro, A. Rickinson, E. Kieff, et al.** 1989. Expression of Epstein-Barr virus transformation-associated genes in tissues of patients with EBV lymphoproliferative disease. N. Engl. J. Med. **321**:1080–1085.